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13. ABSTRACT *(Maximum 200 words)*

Immobilized enzymes are used in bioreactors and biosensors. Current methods for immobilizing enzymes include adsorption or covalent attachment to a support, microencapsulation and entrapment within a membrane/film or gel. The ideal enzyme immobilization method would 1) Employ mild chemical conditions; 2) Allow for large quantities of enzyme to be immobilized; 3) Provide a large surface area for enzyme/substrate contact within a small total volume; 4) Minimize barriers to mass transport of substrate and product and 5) Provide a chemically and mechanically robust system. This report describes a new method for enzyme immobilization that satisfies all of these criteria. We have developed a template-based synthetic method that yields hollow polymeric microcapsules of uniform diameter and length. These microcapsules are arranged in a high density array in which the individual capsules protrude from a surface like the bristles of a brush. We have developed procedures for filling these microcapsules with high concentrations of enzymes. The enzyme-loaded microcapsule arrays function as enzymatic bioreactors in both aqueous solution and organic solvents.

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SYNTHESIS OF POLYMERIC MICROCAPSULE ARRAYS AND THEIR USE FOR ENZYME IMMOBILIZATION

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Immobilized enzymes are used in bioreactors and biosensors (1-20). Current methods for immobilizing enzymes include adsorption or covalent attachment to a support (2-4), microencapsulation (5,6), and entrapment within a membrane/film (7,8,11-20) or gel (9). The ideal enzyme immobilization method would 1) Employ mild chemical conditions; 2) Allow for large quantities of enzyme to be immobilized; 3) Provide a large surface area for enzyme/substrate contact within a small total volume; 4) Minimize barriers to mass transport of substrate and product, and 5) Provide a chemically and mechanically robust system. This report describes a new method for enzyme immobilization that satisfies all of these criteria. We have developed a template-based synthetic method that yields hollow polymeric microcapsules of uniform diameter and length. These microcapsules are arranged in a high density array in which the individual capsules protrude from a surface like the bristles of a brush. We have developed procedures for filling these microcapsules with high concentrations of enzymes. The enzyme-loaded microcapsule arrays function as enzymatic bioreactors in both aqueous solution and organic solvents.

The synthetic methods developed are related to our work on polymeric microtubules (21-24). While such tubules (21-27) are interesting microstructures, they are of doubtful utility for enzyme immobilization because they are microscopic cylinders that are open on both ends. The synthetic strategies described here yield capped versions of such microtubules (i.e. "microcapsules"). Microporous polycarbonate filtration membranes (21-24) were used to prepare these microcapsules. These membranes have cylindrical pores of uniform ($\pm$ 10 %) diameter; these pores are used as templates for preparation of the microcapsules. The membranes used here contained 6×10^8 pores per cm^2, and the pores were 400 nm in diameter. Membranes with significantly higher porosities and with pore diameters as small as 10 nm are available. Hence, microcapsules arrays of the type described here that have nearly any desired capsule density and diameter can be prepared. Finally, the length of the microcapsules obtained is determined by the thickness of the template membrane, in this case 10 μm.

The microcapsule arrays are prepared via a combination of electrochemical and chemical polymerizations. The surface of the template membrane is first sputtered with a ca. 50 nm layer of Au (Figure 1A) which is used to electropolymerize (28) a polypyrrole film across the face of the membrane. Short (1 μm) polypyrrole "plugs" are also deposited within the pores (Figure 1B). Polypyrrole microtubules are then chemically polymerized (21-24) within the pores of the plugged membrane (Figure 1C). The electrochemically-polymerized plugs become caps for the chemically-polymerized tubules.

The capped microtubules are then filled with the desired enzyme by vacuum filtration of a solution of the enzyme through the capsule-containing membrane (Figure 1D). The solvent molecules (H_2O) can pervaporate (29) through the polypyrrole plugs, whereas the much larger enzyme molecules are retained within the capsules. Five enzymes - glucose oxidase, catalase, subtilisin, trypsin, and alcohol dehydrogenase - have been encapsulated and tested to date. After addition of the enzyme, Torrseal epoxy is applied to the upper surface of the membrane (Figure 1E). After curing, the entire assembly is immersed into methylene chloride to dissolve the membrane. This yields the desired array of enzyme-loaded microcapsules (Figures 1G and 2A). A transmission electron micrographic image of capsules that had not been attached to the epoxy surface is shown in Figure 2B. The walls of the capsules are so thin (ca. 25 nm thick) that electrons pass through them. In spite of these thin walls, these capsules have extraordinary mechanical strength (30).

Catalase catalyzes the decomposition of H_2O_2 to O_2 and H_2O (31). Immersion of a catalase-loaded microcapsule array into a solution containing H_2O_2 causes an immediate decrease in the H_2O_2 concentration (Figure 3). When the microcapsule array is removed, H_2O_2 decomposition ceases. That this decomposition is enzyme-mediated is proven by the fact that immersion of a catalase-free capsule array resulted in no change in the H_2O_2 concentration. Finally, these studies show that the enzyme is not leached from the capsules because, if this were the case, the H_2O_2 concentration would continue to decay after the

capsule array was removed from the solution. The issue of enzyme retention will be discussed in greater detail below.

Analogous experiments were conducted with glucose oxidase (GOD, Figure 4A) (32). Curves "a" and "b" in Figure 4A compare catalytic activities for microcapsule arrays containing two different loading levels of GOD. As would be expected, the capsules with the higher GOD-content show higher enzymatic activity. This ability to control the amount of enzyme immobilized is an important feature of this microcapsule immobilization method. An assay (32) of the quantity of GOD loaded into the capsules used for curve "a" showed that an amount equivalent to 625 mg GOD per cm^3 of capsule volume was present in each capsule. It can be shown from the specific volume of GOD (33) that this quantity of GOD occupies 47 % of the available volume within a microcapsule. These data show that high concentrations of enzyme can be loaded into these microcapsules.

Enzyme immobilization is a critical issue in the development of new biosensors. A number of proposed glucose biosensors have been prepared by physically entrapping GOD within polypyrrole films (11-20). Curves "c" and "d" in Figure 4A show the enzymatic activities for two such GOD-containing polypyrrole films (12). The films were ca. 4.7 μm (curve "c") and 0.8 μm (curve "d") thick (14). (The activities for these films are nearly the same because only a thin layer (ca. 0.3 μm-thick) at the outer surface of the polypyrrole film is enzymatically active (13,15).) A comparison of the slopes of curves "c" and "d" with the slope of curve "a" clearly shows that higher enzymatic activity can be achieved with our microcapsule-immobilization method. Hence, our microcapsule arrays show promise for the development of new types of enzymatic biosensor.

There is considerable current interest in the idea of using enzymes in organic solvents (34). Because polypyrrole is insoluble in all solvents, these microcapsule arrays should be compatible with any desired solvent. To demonstrate this, we immersed an array of subtilisin-loaded capsules into dry acetone containing N-acetyl L-phenyl alanine ethyl ester and propanol; subtilisin should catalyze the transesterification reaction. Transesterification is

observed when subtilisin-loaded microcapsules are used and is not observed when the microcapsules are empty (Figure 4B).

Two important questions remain to be addressed - 1) What is the smallest enzyme that can be immobilized? and 2) How large of a substrate molecule will be able to permeate the capsule walls? The enzyme trypsin was used to address the first question. At 23,500 daltons, trypsin, which catalyzes ester hydrolysis, is one of the smallest of enzymes. The catalytic activity of a freshly-prepared array of trypsin-loaded microcapsules was evaluated using Nα-benzoyl-L-arginine ethyl ester as the substrate molecule. The trypsin loaded capsule array was then stored in aqueous buffer solution for 45 days. The catalytic activity was then reevaluated; within experimental error, no loss of enzymatic activity was observed. These data show that trypsin is not leached from these capsules. Again, since trypsin is one of the smallest of enzymes, this means that nearly any enzyme can be permanently immobilized within these microcapsules.

The question of size of the permeant molecule was explored using alcohol dehydrogenase (ADH). ADH catalyzes the oxidation of ethanol using nicotinamide-adenine dinucleotide (NAD) as the electron/proton acceptor. ADH was loaded within the microcapsules and NAD and ethanol were present in the external solution phase. The enzymatic reaction was followed by spectroscopically assaying the external solution for NADH. Experiments analogous to those described in Figures 3 and 4 showed that the ADH-loaded capsules catalyze alcohol oxidation. In order for this to occur both NAD and NADH must permeate the walls of the microcapsules, and these are very large molecules (M.Wt. 660). Because substrate molecules for most enzymatic processes are smaller than this, these experiments show, again, that these tubules can be used with nearly any enzymatic system. Large molecules are able to permeate the capsule wall because polypyrrole is nanoporous (35) and because the walls are extraordinarily thin. Finally, to prove that NAD does indeed permeate the capsule walls, we ran analogous experiments with dextran (M.Wt. 40,000)-linked NAD in the external solution. No enzymatic activity was observed.

We have shown that microcapsule arrays can be prepared via the template synthesis method and that these capsule arrays provide a very general approach for enzyme immobilization. This new enzyme immobilization concept should find applications in biosensors and bioreactors. It is worth mentioning, in closing, that the template method has been used to prepare tubular microstructures composed of a variety of polymers, metals and other materials (22,23). Hence, bioreactors of the type described here that are composed of a variety of different material should be possible.

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Figure Captions.

Figure 1. Schematic diagram of methods used to synthesize and enzyme-load the microcapsule arrays. A. Au-coated template membrane. B. Electropolymerization of polypyrrole film. C. Chemical polymerization of polypyrrole tubules. This is accomplished by immersing the membrane into a solution that contains pyrrole monomer and an oxidizing agent (Fe^{3+}). D. Loading with enzyme. E. Capping with epoxy. The epoxy is too viscous to flood the microcapsules. F. Dissolution of the template membrane.

Figure 2. (A, upper) Scanning electron micrograph of a typical microcapsule array. (B, lower) Transmission electron micrograph of microcapsules that had not been attached to the epoxy surface. Scale bar in upper left corner of B is 1.0 μm.

Figure 3. Evaluation of the enzymatic activity of catalase-loaded capsules. The course of this reaction was followed by monitoring the H_2O_2 absorbance at 240 nm (31). The curve shows absorbance due to H_2O_2 upon insertion (points a, a', a") and removal (points b and b') of a catalase-loaded microcapsule array into a solution that was 35 mM in H_2O_2, pH = 7.0 phosphate buffer.

Figure 4.

(A) Evaluation of the enzymatic activity of glucose oxidase (GOD)-loaded microcapsules (curves "a" and "b"), and empty microcapsules (curve "e"). The standard o-dianisidine/peroxidase assay was used (32). A larger amount of GOD was loaded into the capsules used for curve "a" than in the capsules used for curve "b". Curves "c" and "d" are for a competing GOD immobilization method - entrapment within a polypyrrole film (11-20). All activities have been normalized so that the geometric areas of the polypyrrole films and the geometric area of the membrane used to prepare the array of microcapsules are the same. All data were obtained after immersion of the GOD-containing system (i.e. microcapsule arrays or polypyrrole films) in buffer solution for 12 hours. This was done to remove any GOD that is not permanently immobilized. This is a particular problem for GOD immobilized within the polypyrrole films (11,17).

(B) Evaluation of the enzymatic activity for subtilison-loaded and empty microcapsules. The progress of the transesterification reaction was followed using gas chromatography (34). The solution was 5 mM in ester and 1 M in propanol. The solvent was dry acetone.

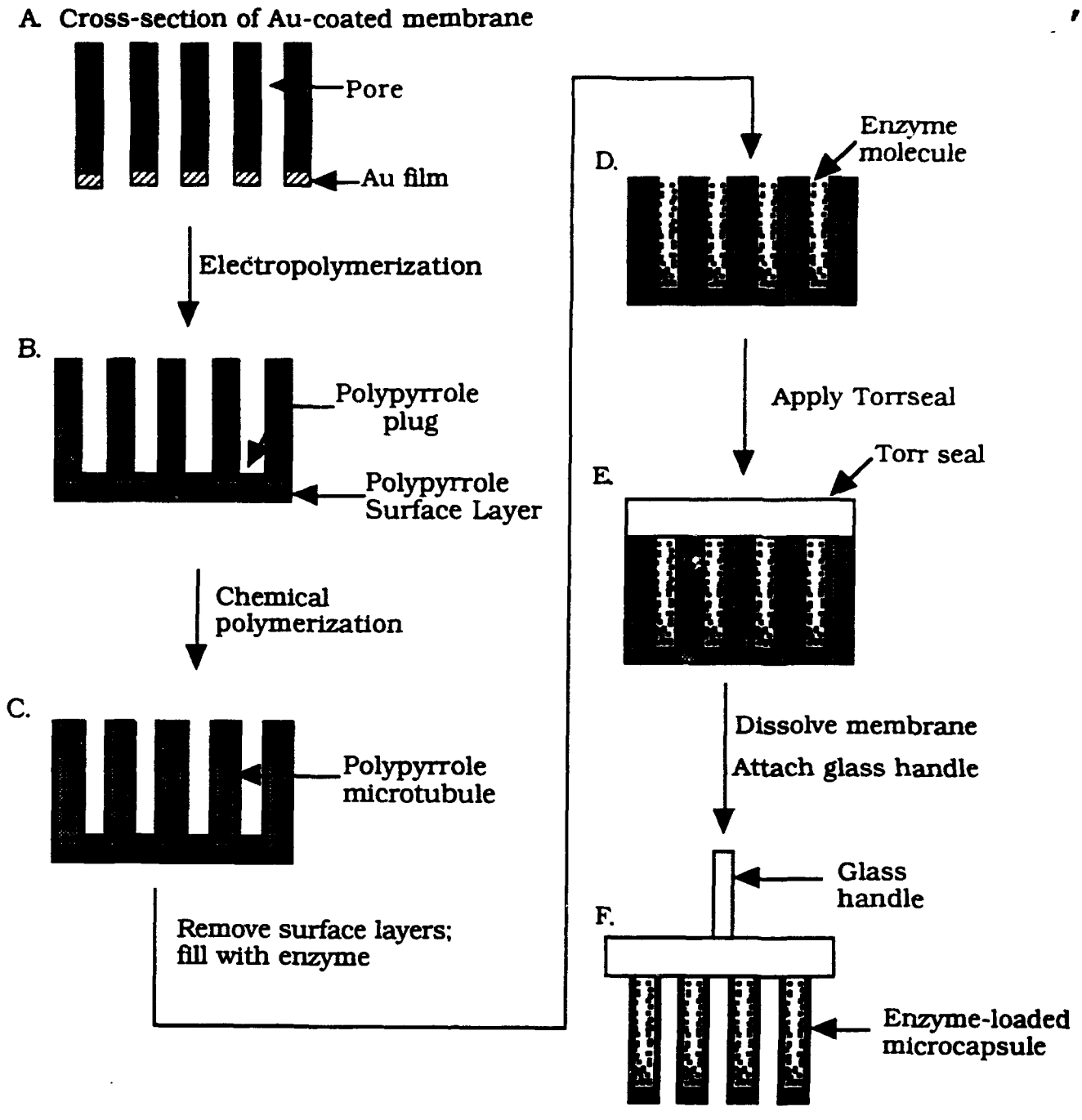
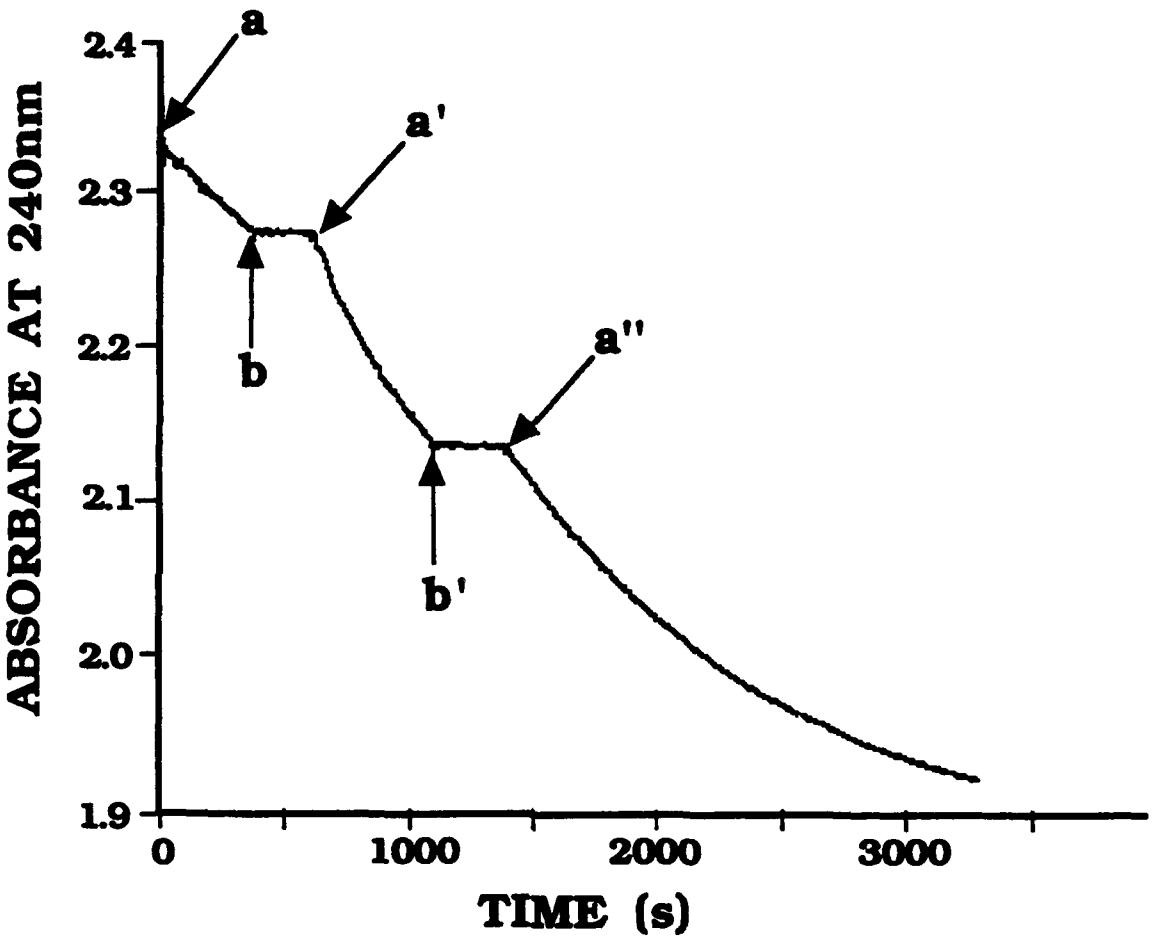

A. Cross-section of Au-coated membrane
Pore
Au film
Electropolymerization
B.
Polypyrrole plug
Polypyrrole Surface Layer
Chemical polymerization
C.
Polypyrrole microtubule
Remove surface layers; fill with enzyme
D.
Enzyme molecule
Apply Torrseal
E.
Torr seal
Dissolve membrane
Attach glass handle
F.
Glass handle
Enzyme-loaded microcapsule

Fig. 1

USE ORIGINALS
PROVIDED WITH ORIGINAL → MANU-SCRIPT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a'
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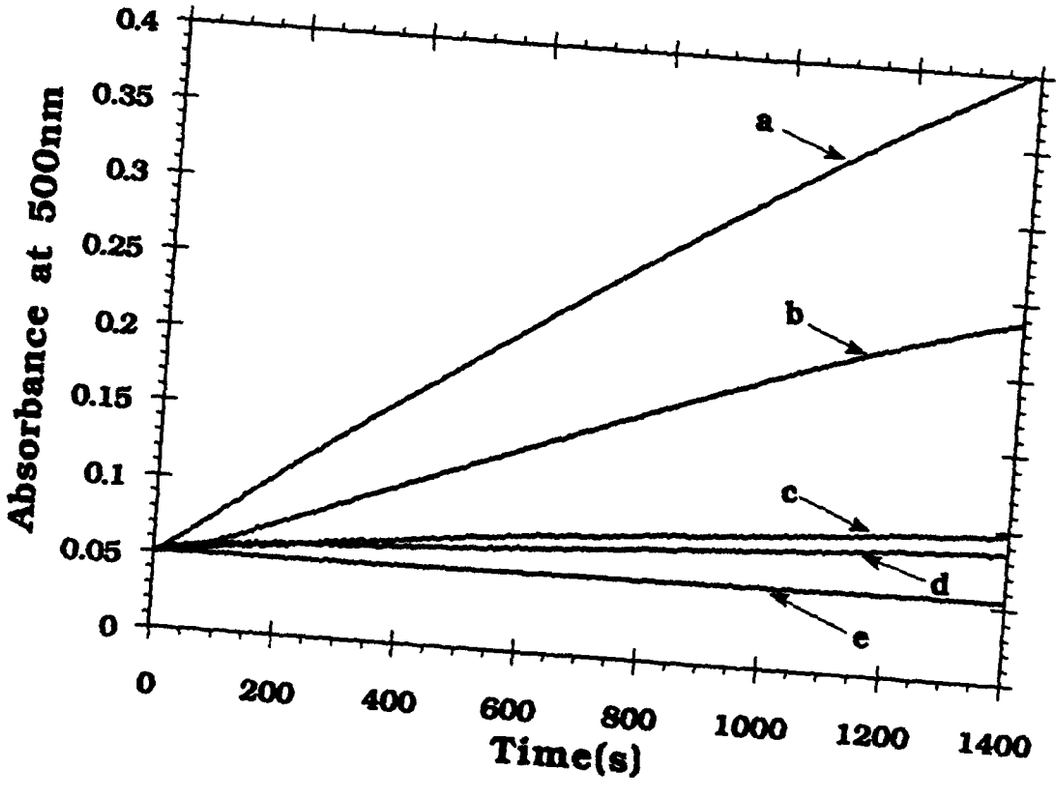
1000
2000
3000
TIME (s)

Fig. 3


Absorbance at 500nm
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1400
Time(s)
a
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Fig 4 A

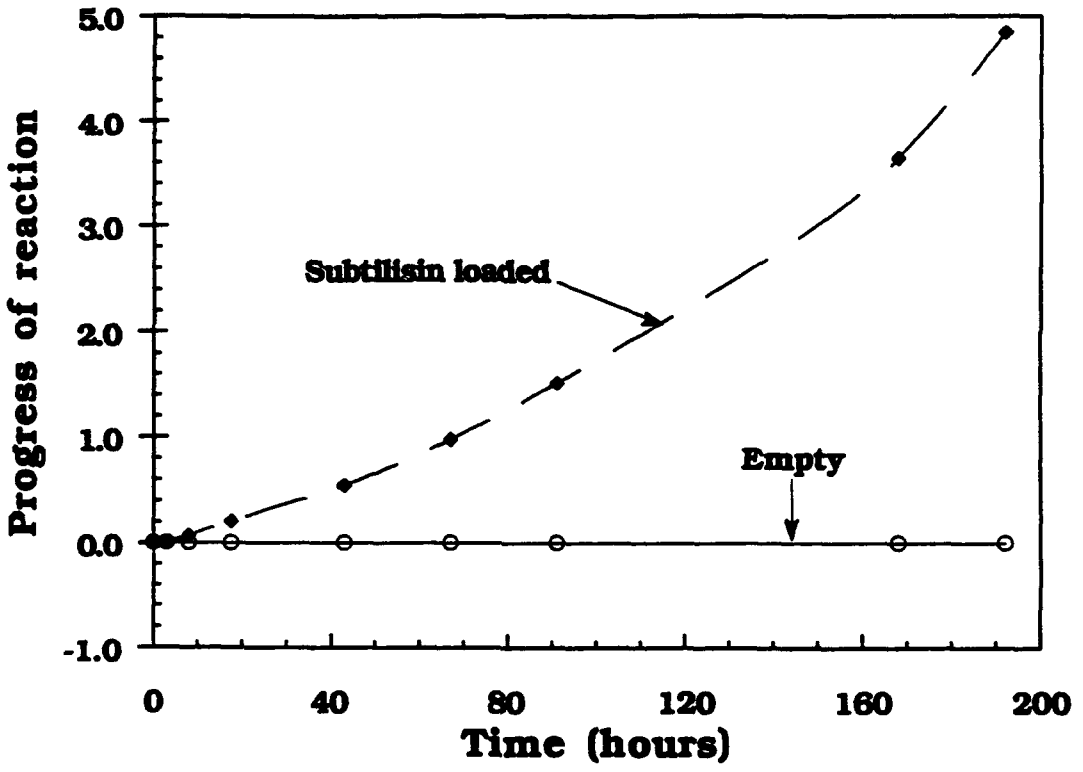
5.0
4.0
3.0
2.0
1.0
0.0
-1.0
Progress of reaction
Subtilisin loaded
Empty
0
40
80
120
160
200
Time (hours)

Fig 1B